MATERIAL AND METHOD

This study was performed at the Immunohematology department, New Civil Hospital, Surat, from June 2012 to February 2014.

Sample size and collection:

The sample size was determined based on the Krejcie and Morgan criteria, for determining the sample size at 95% confidence levels with a margin of error of 5%.\(^{[162]}\)

In last few years, every year around seven thousand five hundred blood donors between 18 and 60 years have been volunteering at the blood bank for blood donation. Krejcie and Morgan in their criteria of sample selection have suggested a minimum sample of 365 against the 7500 population at 95% confidence level (p <0.05). The present study includes total 393 samples.

The sample size table freely available on the website www.research-advisors.com/tool/samplesize.htm.

Table: Sample size requirement guideline.
Total 393 random voluntary blood donor samples collected and tested for various tests to evaluate the iron status of the donors. Voluntary donors were those who donated blood without remuneration or any relative to donate. Each donor donated 300 – 450 mL of whole blood depending on their body weight.

The eligible donors were divided into two main groups like first time donors and repeat donors. The second repeat donor group was subdivided into different subgroups like as
Iron status in voluntary blood donors

per their last donation into group I to IV, and as per their frequency of donation into group A to D.

<table>
<thead>
<tr>
<th>Donors</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 - 5</td>
<td>6 - 9</td>
<td>10 - 20</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Months</td>
<td>Months</td>
<td>Months</td>
<td>Months</td>
<td></td>
</tr>
<tr>
<td>Group A [1-5 times]</td>
<td>42</td>
<td>52</td>
<td>25</td>
<td>6</td>
<td>125</td>
</tr>
<tr>
<td>Group B [6-10 times]</td>
<td>28</td>
<td>42</td>
<td>12</td>
<td>2</td>
<td>84</td>
</tr>
<tr>
<td>Group C [11-20 times]</td>
<td>12</td>
<td>25</td>
<td>7</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>Group D [&gt;20 times]</td>
<td>12</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>94</td>
<td>134</td>
<td>45</td>
<td>9</td>
<td>282</td>
</tr>
</tbody>
</table>

Methods

The present study was duly approved by Human Research ethics committee of Government, Medical College, Surat.

Donors were selected on the basis of the criteria laid down in the Technical Manual of the Directorate General of Health Services, Ministry of Health, Government of India for blood donation.\[163\]

The selected donors were asked specific questions as per donor questionnaire, i.e., age, total number of life time donations, date of last donation/previous donation, dietary history; vegetarian and non-vegetarian food intake. All selected donors were healthy
Iron status in voluntary blood donors according to their clinical histories and physical examinations and fulfilled the suitability criteria for donation. The hemoglobin levels were screened using hemoglobin testing by copper sulphate specific gravity method \cite{[164]} and only donors with hemoglobin of more than 12.5 g/dl were allowed to donate. Informed written consent for blood donation and blood testing was obtained from each donor who fulfilled the criteria for blood donation by the staff of blood bank before collecting blood for donation.

**Sample Inclusion Criteria :-**

- Voluntary blood donors including regular donors as well as first time donors.
- Any age ( fitted to donor collection criteria)
- Any sex

**Sample Exclusion criteria:-**

- Voluntary donors who found to have increased ESR level as a marker for any hidden chronic infection which may affect the results of iron study parameter.
- Persons receiving iron therapy.

After blood donation of whole blood, an additional sample was taken via the bleed line into a 2.5 mL EDTA tube for assays of complete blood count, ZPP and ESR estimation and into 4.0 mL plain tubes for measurement of serum iron, serum ferritin, and total iron binding capacity. Serum was separated from plain samples collected in the pilot tubes from each donor and stored at -40 °C. Complete blood count was performed on three part hematology analyzer and ESR was performed by modified Westergren’s method. That will rule out the presence of
any chronic inflammatory disorder. ZPP was performed by using Helena protoFluor reagent system on hematofluorometer. Serum Iron, TIBC and serum ferritin tests were performed in biochemistry department with the help of their staff. Serum ferritin was measured by standard enzyme immunoassay technique and serum iron and TIBC done by photometric test using chromogen ferrozine. Transferrin saturation was calculated from the serum iron concentration and TIBC values as follows: transferrin saturation = serum iron/TIBC x 100. The assays were validated using appropriate controls for each lot used.

In all donor samples the Mentzer index is applied on count values. The Mentzer index is defined as mean corpuscular volume per red cell count. An index of less than 13 suggests that the patient has the thalassemia trait, and an index of more than 13 suggests that the patient has iron deficiency. In all samples with the Mentzer index value < 13, a further diagnostic test HPLC is performed to differentiate between iron deficiency anemia and beta thalassemia trait as a diagnosis. HbA2 quantitation was done by HPLC on an automated system (Bio Rad Variant II, D-10, Bio-Rad Laboratories, CA). Diagnosis of IDA was made based on plasma ferritin values lower than 15 ng/ ml. Diagnosis of BTT was made based on HbA2 levels more than 3.5%.

Data were analyzed in excel sheet and using statistical computer software. Comparisons between populations were made using the mean, standard deviation, t-test, $\chi^2$ test and Anova test. An alpha value of < 0.05 denoted a statistically significant difference.
Ferritin values below 15 µg/L were considered iron deficient, 15-20 borderline, and above 20 normal. Hemoglobin values below 12.5 g/100 ml were considered to represent anemia.

If any donors with low MCV have normal iron status then further investigation to rule out possibility of thalassemia trait has been performed as and when required.

**Analysis of various hematological parameters by three part cell counter:**

**Principle:**

The impedance method counts & sizes cells by detecting & measuring changes in electrical impedance when a particle in a conductive liquid passes through a small aperture.

**Calibration procedure:**

Calibration is done by using known calibrator material at regular time interval.

**Quality control procedure:**

Various internal and external quality control policies exist in the lab and that is followed regularly.

**ESR test by Modified Westergren’s method:**

**Principle:**

When well mixed venous blood is placed in a vertical tube, erythrocyte will tend to fall towards the bottom. The length of fall of the top of the column of erythrocytes in a given time interval is called ESR.

**Procedure:**
Westergren tube is 300mm in length, bore size 2.55mm. ESR cup is filled with 0.5ml sodium citrate and then 2ml whole blood from EDTA vacutte added. Put the Westergren pipette in the ESR cup and is filled up to 0 mark. Pipette placed vertical in the rack at R.T. After 60 minute distance from 0 mark to top of the column of red cells is recorded in mm as ESR value.

Reference range for males <10 mm/hr and for females <20 mm/hr.

**Serum Iron test:**

**Method:**

Photometric test using chromogen ferrozine.

**Principle of examination:**

Transferrin-bound iron is released at an acidic pH and reduced from ferric to ferrous ions with reducing substances (like ascorbic acid, hydroxylamine). These ions react with ferrozine to form a violet colored complex which is measured spectrophotometrically at 560 nm. The absorbance measured at this wavelength is proportional to serum iron concentration. Thiosemicarbazine is used to prevent complex of Cu²⁺ with Ferrozine. Iron exists in serum complexed with transferrin, a transport protein. Most early procedures for iron determination involved dissociation of the iron from the iron-protein complex precipitation of the proteins, and the measurement of the iron content of the protein free filtrate.

Many chromogens have been used in the determination including thiocyanate o-phenantroline, bathophenantroline and TPTZ. In 1971 Presijn et al presented a method using the chromogen ferrozine, described by Stookey. This method did not require
protein precipitation and was more sensitive than previous methods. The present procedure is a modification of the Presijn method.

**Total Iron Binding Capacity Examination in serum:**

**Principle of examination:**

A known amount of ferrous ions are added to serum at an alkaline pH. The ferrous ions bind with transferrin at unsaturated iron binding sites. The additional unbound ferrous ions are removed by adsorbed with Mg2CO3. Iron is measured from supernatant sample after centrifugation. The TIBC is equal to the serum iron concentration of Supernatant sample.

**Ferritin test:**

**Principle:**

The ferritin assay is based on simultaneous binding of human ferritin to two monoclonal antibodies; one is immobilized on the microplate, the other is soluble and conjugated with horseradish peroxidase (HRP). Microtiter strip wells are precoated with anti- ferritin IgG antibodies. Ferritin in samples and standards binds to the immobilized antibodies on the surface of the microtiter wells and the second, soluble anti-ferritin antibody conjugated with HRP binds to the immobile antibody-ferritin –complex during the first incubation. Afterwards a bound/free separation is performed by solid-phase washing. The immune complex is visualized by adding tetramethylbezidine substrate, which gives a blue reaction product. The intensity of this product is proportional to the amount of ferritin in samples and standards. Sulphuric acid is added to stop the reaction. This produces a
yellow endpoint colour. Absorption at 450 nm is read using an ELISA microwell plate reader.

**Zinc protoporphyrin:**

It was measured by the Helena ProtoFluor Reagent system designed for use with a hematofluorometer. It is a very simple, easy, accurate and inexpensive method for determining erythrocyte ZPP levels in the diagnosis of iron deficiency. The method requires only one drop of whole blood and no sample measurements are necessary.

The principle of hematofluorometry for measuring zinc protoporphyrin requires that the hemoglobin be fully oxygenated. By adding Protofluor reagent to whole blood, the hemoglobin is derivitised to a product having the spectral characteristics of oxyhemoglobin in the region where the hematofluorometer operates. Thus, the need for oxygenation is circumvented which allows the determination of zinc protoporphyrin with greater accuracy and precision, even in moderately aged and deoxygenated blood.

**Method:**

1] Take one drop of the patient whole blood sample in a small test tube.

2] add two drops ProtoFluor Reagent.

3] Mix by shaking briefly [a few seconds is adequate].

4] Pour a drop of specimen onto a glass cover slip which has been placed into the sample holder. Spread the drop using the lip of the test tube so that the specimen covers the appropriated area of the cover slip.

5] Proceed with the measurement process. Once the blood and reagent have been mixed, the solution is stable for up to 5 minutes. After a reading has been taken, the test mixture
Iron status in voluntary blood donors is no longer stable. Irradiation of the blood sample during reading may cause photodecomposition making subsequent measurements from the same sample unreliable.

**HPLC – Biorad Beta Thal Variant System**

**Principle**
HPLC depends on the interchange of charged groups on the ion exchange material with charged groups on the haemoglobin molecule. A typical column packing is 5 μm spherical silica gel. The surface of the support is modified by carboxyl groups to have a weakly cationic charge, which allows the separation of haemoglobin molecules with different charges by ion exchange. When a haemolysate containing a mixture of haemoglobins is adsorbed onto the resin, the rate of elution of different haemoglobins is determined by the pH and ionic strength of any buffer applied to the column. Elution of the charged molecules is achieved by a continually changing salt gradient; fractions are detected as they pass through an ultraviolet/visible light detector and are recorded on an integrating computer system. Analysis of the area under these absorption peaks gives the percentage of the fraction detected. The time of elution (retention time) of any normal or variant haemoglobin present is compared with that of known haemoglobins, providing quantification of both normal haemoglobins (A, F, and A₂) and many variants.